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European Journal of Pharmacology 294 (1995) 547–553

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σ_2 Site-mediated inhibition of electrically evoked guinea pig ileum longitudinal muscle/myenteric plexus contractions

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Received 1 May 1995; revised 13 September 1995; accepted 14 September 1995

Abstract

Functional and binding studies were performed in order to characterize the relative efficacy and affinity of a number of compounds that bind to σ sites. The ability of σ site ligands to inhibit electrically evoked contraction of the guinea pig ileum longitudinal muscle/myenteric plexus preparation was compared to the affinities of these compounds for σ_1 sites (assessed by displacement of [^{3}H](+)-pentazocine) and σ_2 sites (assessed by displacement of [^{3}H]1,3-di-*o*-tolylguanidine (DTG) in the presence of 5 μM dextromethorphan). It was shown that the rank order of potencies for suppression of electrically evoked contractions of guinea pig ileum perfectly matched the rank order of affinities of these compounds for the σ_2 binding site, while correlating poorly with the σ_1 binding site. In addition, no significant correlations were found between the efficacy of the tested compounds to inhibit contraction of the guinea pig ileum preparation and previously reported affinities for muscarinic, dopamine D_2 or MK-801 binding sites. Thus, the present study represents the first functional bioassay selectively sensitive to agents interacting with the σ_2 receptor subtype binding site, and provides a means with which to further elucidate the functional role of σ_2 sites.

Keywords: σ Binding site; Smooth muscle; Ileum; σ Receptor subtype; (Guinea pig); (Rat)

1. Introduction

σ Binding sites were first postulated in 1976 as a subtype of opiate receptor (Martin et al., 1976). σ Sites were later identified in the guinea pig brain using radiolabeled *N*-allylnormetazocine (SKF-10,047) (Su, 1982), and more recently using haloperidol (Contreras et al., 1987), 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ((+)-3-PPP) (Gundlach et al., 1986), *N*,*N*′-di(*o*-tolyl)guanidine (DTG) (Weber et al., 1986), and (+)-pentazocine (De Costa et al., 1989). These studies have found that σ sites are widely distributed throughout the brain and periphery leading to speculation that this system may play a role in psychosis (Su, 1993; Walker et al., 1990), motor dysfunction (Matsumoto et al., 1989, 1990; Walker et al., 1990), emetic processes (Hudzik, 1991), and hepatic (Musacchio et al., 1988; Walker et al., 1990), endocrine (Su et al., 1988; Walker et al., 1990; Wolfe et al., 1988), and intestinal functions (Campbell et al., 1989). However, relatively little is actually known about the functional significance of this binding site.

Some of the confusion surrounding the functional significance of σ binding sites may, in part, stem from the existence of at least two (Gundlach et al., 1986; Hellewell and Bowen, 1990; Vu et al., 1990; Walker et al., 1990; Wolfe et al., 1988, 1989) and possibly three (Meyers et al., 1994), subtypes of σ binding sites. A second σ binding site was first identified in PC12 cells, a cell line derived from the rat adrenal medulla (Bowen and Hellewell, 1988). In this study it was shown that σ site ligands showed affinities for a σ site in a rank order of potency which differed from the profile seen in the guinea pig brain (Bowen and Hellewell, 1988). These sites were later classified as σ_2 binding sites (Hellewell and Bowen, 1990). It was soon shown that σ_1 and σ_2 sites exist in both the brain and periphery in a wide variety of species (Gundlach et al., 1986; Vu et al., 1990; Wolfe et al., 1988, 1989). Although both σ_1 and σ_2 sites possess high affinity for haloperidol and DTG, there are several differences. σ_1 Sites are enan-

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SSDI 0014-2999(95)00584-6

tioselective for (+)-benzomorphans, whereas σ_2 sites are enantioselective for (−)-benzomorphans (Bowen and Hellewell, 1988; Hellewell and Bowen, 1990). Further, σ_1 and σ_2 sites occur as proteins with different molecular weights (approximately 25 kDa and 18–21 kDa, respectively) (Hellewell and Bowen, 1990). Finally, σ_1 and σ_2 sites have different anatomical distribution among species. For example, there is a higher concentration of σ_2 compared to σ_1 sites in the rat liver than in the guinea pig brain (Hellewell et al., 1990).

Functionally, it has been shown that electrically induced contractions of the guinea pig vas deferens are potentiated by compounds with a high affinity for the σ site, with (−)-opiates acting more potently than (+)-opiates which is indicative of σ_2 site mediation (Vaupel and Su, 1987). However, these results appear at conflict with the results of binding studies using vas deferens tissue, which showed a σ_1-like binding profile (Su and Wu, 1990). Campbell et al. (1989) have shown that σ site ligands decrease contractions of the guinea pig ileum longitudinal muscle/myenteric plexus preparation induced by electrical stimulation or application of 5-hydroxytryptamine agonists via a non-cholinergic mechanism. While binding data were presented in this latter report, no data were presented as to the relative affinities of the compounds tested for the σ_1 versus σ_2 site subtype. Given the clear differences between these two subtypes, they may subserve very different functions physiologically. Thus, the present study examined the effects of a variety of σ site ligands on electrically induced contractions of the guinea pig ileum longitudinal muscle/myenteric plexus preparation. In addition, the relative affinity of these compounds for both σ_1 and σ_2 binding sites was assessed.

2. Materials and methods

2.1. σ Receptor binding assay

Binding assays were performed using a modification of previously described methods (De Costa et al., 1989; Bowen et al., 1988, 1990). Briefly, frozen male Hartley guinea pig (Charles Rivers, MA, USA) brains with the cerebella removed were thawed at room temperature. All procedures were conducted at 4°C unless otherwise noted. The brains were homogenized in ice-cold 0.32 M sucrose in 10 mM Tris-Cl (pH 7.4) with a motor-driven Teflon-glass homogenizer in a volume of 10 ml/g of tissue. The homogenate was centrifuged at $1000 \times g$ for 10 min in a refrigerated centrifuge. The supernatant was removed and the pellet was resuspended in ice-cold 0.32 M sucrose, 10 mM Tris-Cl (pH 7.4, 5 ml/g) and again centrifuged at $1000 \times g$ for 10 min. The supernatants were combined and centrifuged at $31\,000 \times g$ for 15 min. The pellet was resuspended in 10 volumes of 10 mM Tris-Cl (pH 7.4) and allowed to incubate at room temperature for 15 min followed by centrifugation at $31\,000 \times g$ for 15 min. The pellet was then resuspended in 10 volumes of 5 mM Tris-Cl (pH 8.0). Just prior to the assay, the suspension was homogenized for 30 s. The σ_1 assay was carried out in a total volume of 0.5 ml, consisting of 5 mM Tris-Cl (pH 8.0), 1–2 nM [^{3}H](+)-pentazocine (15–30 Ci/mM, Dupont), 0.1 ml of membrane suspension (1.5–2.0 mg protein), and various concentrations of the test compounds.

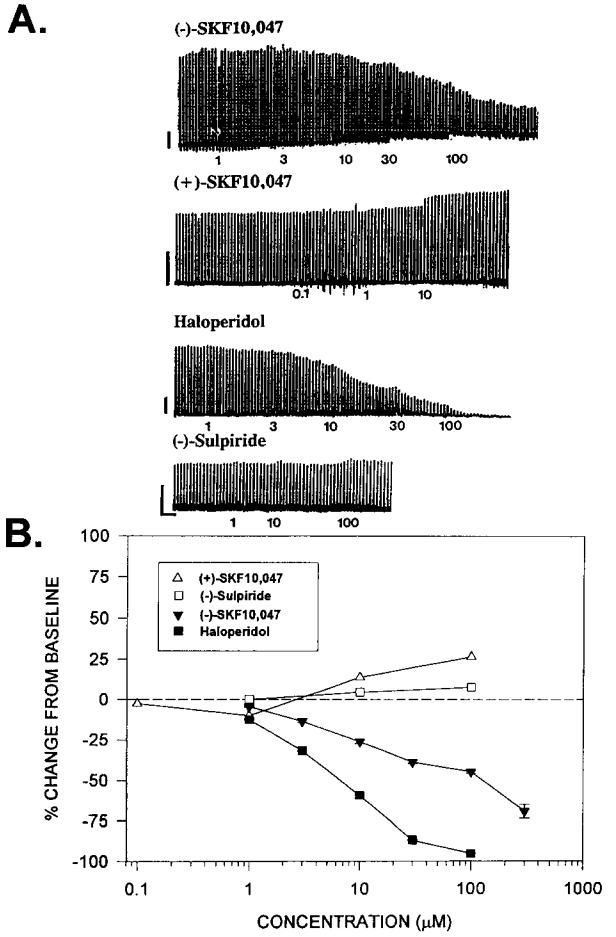

Fig. 1. A: Effects of (−)-SKF10,047, (+)-SKF10,047, haloperidol and (−)-sulpiride, respectively, on electrically evoked contraction of guinea pig ileum longitudinal muscle/myenteric plexus preparation. Compare the concentration-dependent decreases in electrically evoked guinea pig ileum longitudinal muscle/myenteric plexus contractions produced by (−)-SKF10,047 and haloperidol with the lack of effect of (+)-SKF10,047 and (−)-sulpiride. Scale bars: vertical = 0.5 g; horizontal = 1 min. Numbers at the bottom of the tracings represent the concentration (μM) of the given compound. B: Effects of (+)-SKF10,047, (−)-sulpiride, (−)-SKF10,047 and haloperidol on electrically evoked guinea pig ileum longitudinal muscle/myenteric plexus contractions. Error bars represent standard error of the means.

Nonspecific binding was determined by including 100 μM (+)-pentazocine. Specific binding was determined by subtracting the dpm bound in the presence of 100 μM (+)-pentazocine from that bound in the absence of added (+)-pentazocine or test compound. After 16–18 h of incubation at room temperature, the membranes were collected onto a Whatman GF/B filter (Brandell M-48 Harvester), which was presoaked overnight in 0.3% polyethylenimine. The assay tubes and filters were rinsed 2 times with 4–5 ml cold 5 mM Tris-Cl (pH 8.0) and the filters were counted in a scintillation counter after soaking in Ecolume (ICN) scintillation fluid for 16–18 h. Protein was measured with a BCA protein reagent assay kit (Pierce Chemical Co.), using bovine serum albumin as the standard.

Affinity for σ_2 sites was measured in the same way as described for [^{3}H](+)-pentazocine binding, except male Sprague-Dawley rat (Harlan, WI, USA) liver membrane prepared from fresh liver was used as the receptor source, and [^{3}H]DTG (25–30 Ci/mM, Dupont) was used as the ligand. The reaction was carried out in 10 mM Tris-Cl (pH 7.4), with dextromethorphan (5 μM) included to mask σ_1 sites.

IC_{50} values, 95% confidence intervals, and standard error of the means (S.E.M.) were obtained using a nonlinear curve fitting program (Graph Pad). All binding experiments (1–6 experiments per compound) were performed in triplicate for each compound tested.

2.2. Guinea pig ileum longitudinal muscle / myenteric plexus functional assay

Sections of guinea pig ileum longitudinal muscle/myenteric plexus were prepared as described by Campbell et al. (1989). Briefly, an 8–10 cm section of small intestine 10–25 cm proximal to the ileocecal junction was removed from male Hartley guinea pigs (200–300 g, Charles Rivers, MA, USA) following killing of the animal by overexposure to CO_2. The removed intestine was immediately placed in a Krebs solution with the following composition (mM): NaCl, 118; KCl, 4.75; $CaCl_2$, 2.54; $MgCl_2$, 1.17; $NaHCO_3$, 25.0; KH_2PO_4, 0.93; dextrose, 11.0. Sections of intestine approximately 1–2 cm in length were gently stretched over a moistened glass rod and a longitudinal cut was made with a scalpel blade. The longitudinal muscle/myenteric plexus was gently removed using a cotton-tip applicator moistened with the Krebs solution. The strips were placed in 20 ml organ baths containing the Krebs solution at 37.0–37.5°C and bubbled with a 5% CO_2 and 95% O_2 gas. The strips were placed under a 1 g load and contractility was measured using Grass FT03 transducers connected to a Gould 2600S chart recorder. The strips were stimulated at 0.05 Hz using a Grass dual channel stimulator (S88) set at maximal voltage (≈ 150 V, 1–10 ms in duration). Platinum concentric electrodes positioned above and below the strips delivered the stimulation current. Following a 90–120 min equilibrium period during which the bath was changed several times, test compounds were administered in a cumulative fashion, allowing a minimum of 5 min before additional compound was added to the bath. The concentration-effect curves of (−)-pentazocine and (−)-SKF10,047 were determined in the presence of 10 μM naloxone due to the affinity of these compounds for μ-opioid receptors. The effectiveness of a given compound to inhibit electrically induced contraction was measured as the percent change from baseline conditions. The concentration of a given test compound to produce a half-maximal inhibition of the electrically induced contraction (IC_{50}) was determined with a nonlinear curve-fit program (Interactive Statistical System) using the mean response of at least 3 separate trials as the given response for a single concentration. The correlations between IC_{50} values to inhibit electrically induced contractions and affinities

Table 1
Efficacy and affinity values for various σ ligands (±S.E.M.)

Compound	σ_1 Site binding IC_{50} (nM)	σ_2 Site binding IC_{50} (nM)	Inhibition of GPLMMP contraction IC_{50} (μM)	[a] Musc. Ach. binding (nM)	[a] PCP binding (nM)	[a] Dopamine D_2 binding (nM)
1. DTG	137.0 ± 5	100.0 ± 1.7	5.267 ± 0.38	3600 [c]	12000 [d]	> 100000 [e]
2. BMY-14802	300.0 ± 22	131.0 ± 13.6	5.282 ± 0.46	32100 [f]	> 200000 [f]	4000 [f]
3. Haloperidol	3.5 ± 0.1	322.7 ± 33	5.687 ± 0.46	2700 [c]	5000 [g]	1.9 [h]
4. (−)-Pentazocine	251.0 ± 1.7	345.0 ± 26	5.98 ± 0.7 [b]	2500 [c]	N.T.	N.T.
5. Rimcazole	7360.0 ± 2830	2740.0 ± 361	6.253 ± 0.48	27000 [f]	> 200000 [f]	86000 [f]
6. (+)-Pentazocine	3.4 ± 0.05	4077.5 ± 155	16.748 ± 0.68	800.0 [c]	1900 [i]	> 100000 [j]
7. (−)-SKF10,047	8060.0 ± 3280	7763.0 ± 150	19.209 ± 4.8 [b]	N.T.	5650 [j]	14000 [h]
8. Dextromethorphan	577.3 ± 5.4	14300 ± 1180	42.158 ± 0.53	N.T.	2500 [j]	N.T.
9. (+)-SKF10,047	218.0 ± 13.5	54633 ± 2300	> 100	N.T.	1170 [j]	29000 [h]
10. (−)-Sulpiride	> 1000000	> 1000000	> 100	87000 [c]	N.T.	301.0 [h]

[a] K_i or IC_{50}. [b] Tested in the presence of 10 μM naloxone. [c] Bowen et al. (1992). [d] Contreras et al. (1988). [e] Coccini et al. (1991). [f] Largent et al. (1988). [g] Meltzer et al. (1992). [h] Tam and Cook (1984). [i] Iyengar et al. (1990). [j] Murray and Leid (1984). N.T. = not tested or not found in literature.

for binding sites were assessed using a rank correlation test and/or the Pearson product-moment correlation test.

2.3. Drugs

(+)-*N*-Allylnormetazocine ((+)-SKF10,047), (−)-*N*-allylnormetazocine ((−)-SKF10,047) and rimcazole · HCl were obtained from Research Biochemicals International, Natick, MA, USA. Haloperidol, 1,3-di-*o*-tolylguanidine (DTG) and (−)-sulpiride were obtained from Sigma Chemical Co., St. Louis, MO, USA. (+)-Pentazocine and (−)-pentazocine were synthesized in the Chemistry Department of Fisons Pharmaceuticals (now Astra Arcus USA), Rochester, NY, USA. Dextromethorphan and (±)-α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine-butanol (BMY-14802) were generous gifts from Roche Chemicals and Bristol-Myers Squibb, respectively. With the exception of DTG, all compounds were dissolved in sterile H_2O and delivered at the concentrations indicated. DTG was first dissolved in a minimal amount of dilute lactic acid, brought to a pH of 7.0 using dilute NaOH, and brought to volume with H_2O.

3. Results

With the exception of (+)-SKF10,047 and (−)-sulpiride, all of the compounds tested produced concentration-dependent inhibition of electrically induced guinea pig ileum longitudinal muscle/myenteric plexus contractions (Table 1 and Fig. 1). The most potent compounds (DTG, BMY-14802, haloperidol and (−)-pentazocine) were compounds with high affinity for the σ_2 binding site (IC_{50} values = 0.1–0.345 μM), but not necessarily high selectively over the σ_1 site. The ratios of σ_2/σ_1 binding for DTG, BMY-14802, haloperidol and (−)-pentazocine were 0.73, 0.44, 92.2 and 1.38, respectively. The rank order of compounds to inhibit

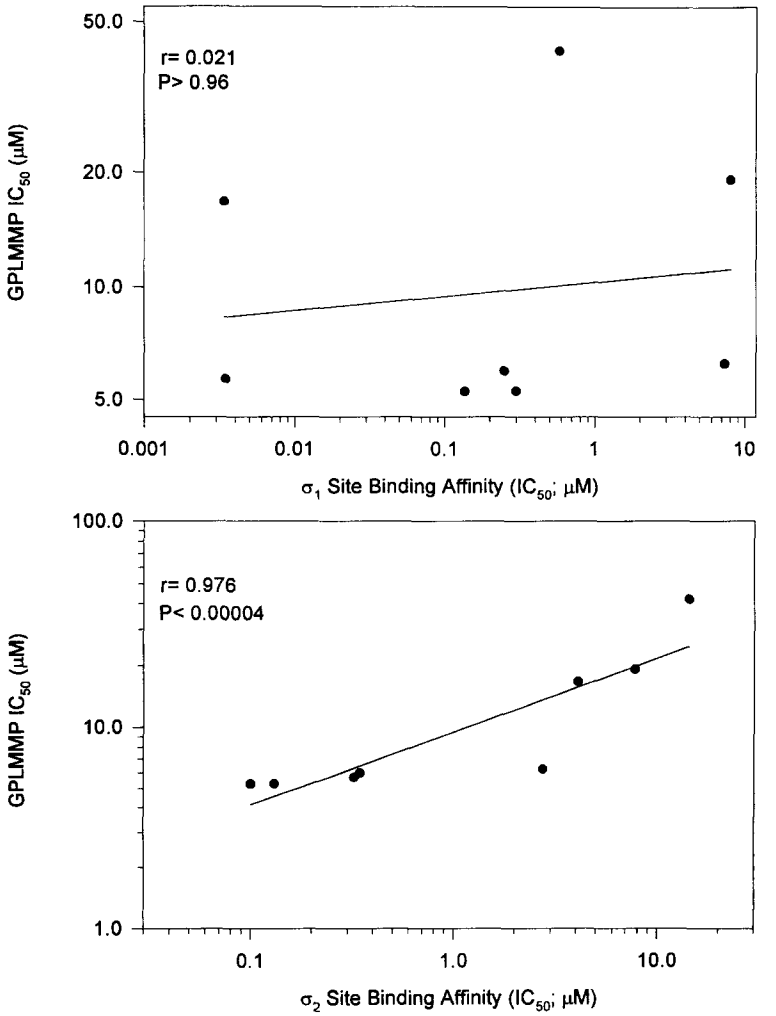

Fig. 2. Correlation of binding affinities for the σ_1 (top panel) and σ_2 (bottom panel) binding sites and efficacy in the guinea pig longitudinal muscle/myenteric plexus preparation assay (GPLMMP) for tested compounds (see Table 1).

guinea pig ileum longitudinal muscle/myenteric plexus contractions was perfectly correlated with their rank affinities for the σ_2 ($r = 1.0$), but not the σ_1 ($r = 0.283$, $P > 0.46$) binding site. Additional analysis, using previously reported binding affinities (Table 1), revealed no significant correlations between rank efficacy to inhibit guinea pig ileum longitudinal muscle/myenteric plexus contractions and rank affinity for muscarinic acetylcholine ($r = 0.036$, $P > 0.939$), D_2 dopamine ($r = -0.181$, $P > 0.669$), or MK-801 ($r = -0.577$, $P > 0.175$) binding sites.

Fig. 2 compares the correlations between binding affinity for σ_1 (top panel) or σ_2 (lower panel) sites and activity in the guinea pig ileum longitudinal muscle/myenteric plexus assay. As seen in Fig. 2 and quantified using the Pearson product-moment correlation, there is no significant relationship between σ_1 site binding and guinea pig ileum longitudinal muscle/myenteric plexus activity ($r = 0.021$, $P > 0.96$), whereas this relationship for σ_2 site binding and guinea pig ileum longitudinal muscle/myenteric plexus activity is extremely significant ($r = 0.976$, $P < 0.000034$).

4. Discussion

The results show that the efficacy of σ site ligands to inhibit electrically induced contractions of the guinea pig ileum is highly correlated with affinity for the σ_2, but not σ_1 site subtype. The lack of correlation to previously reported muscarinic acetylcholine receptor binding indicates that the effects of the σ site ligands tested were neither due to non-selective blockade of receptors on smooth muscle nor activation of muscarinic receptors on myenteric cholinergic neurons. This latter finding is in agreement with Campbell et al. (1989), in which a variety of σ binding site ligands produced no alterations on the inhibitory effect of bethanechol in this preparation. The lack of inhibition by (−)-sulpiride and MK-801 (data not shown) further underscores the lack of involvement of dopamine and MK-801 receptor sites, respectively, in this assay. Also in agreement with Campbell et al. (1989), we found that (+)-SKF10,047 occasionally produced an increase in electrically induced contractility. The mechanism responsible for this increase remains unclear.

To our knowledge this is the first demonstration of a functional in vitro assay with the ability to selectively identify actions at the σ_2 binding site, providing a starting point from which to assess the functional characteristics of this system. As such, these data have vast implications in future research into the etiology of numerous disorders which have been linked to σ binding sites. In addition, these results represent the first evidence that guinea pig intestinal motility is specifically modulated by a σ_2 mechanism.

These studies further indicate that caution should be used when attempting to assign a σ based mechanism to a functional assay. For example, the choice of [^{3}H]DTG or [^{3}H]haloperidol for affinity studies may be less than ideal since both bind with relatively high affinity to both σ_1 and σ_2 sites. Thus, any conclusions based on such binding studies, which attempt to link σ site ligands to biological function, may prove inadequate. This is illustrated in that there are no significant correlations (using Pearson product-moment correlational analyses) between the efficacy of the compounds tested in the present study to inhibit guinea pig ileum longitudinal muscle/myenteric plexus contraction and the K_i values of these compounds to displace [^{3}H]DTG, [^{3}H]pentazocine, [^{3}H](+)-PPP or [^{3}H](+)-SKF10,047 in the guinea pig brain ($P > 0.88$, $P > 0.72$, $P > 0.88$, $P > 0.82$, respectively), as reported in previous studies (see Walker et al., 1990 for review). However, when σ_1 and σ_2 binding was differentially determined, as in the present study, the efficacy of these compounds is strongly correlated with affinity at the σ_2 subtype. Thus, it appears that additional analysis of past studies may reveal specific modulation of functional systems by specific σ subtypes. In the only previous study to examine the relationship between σ ligand binding and guinea pig ileum longitudinal muscle/myenteric plexus efficacy (Campbell et al., 1989), a very low overall correlation was found (using least squares linear regression) between displacement of [^{3}H]DTG in the guinea pig brain and the efficacy of all tested compounds to inhibit electrically stimulated guinea pig ileum longitudinal muscle/myenteric plexus contractions ($r = 0.37$) (Campbell et al., 1989; Walker et al., 1990). This low correlation may have reflected the fact that modulation of the guinea pig ileum longitudinal muscle/myenteric plexus bioassay is linked to a compound's affinity for the σ_2 binding site, and not the σ_1 binding site. As such, the development of new functional assays will require careful examination of the affinity of test compounds for specific σ site subtypes. Development of such σ subtype specific assays is crucial for forwarding investigations into the etiology of disease states in which σ sites have been implicated.

One such condition, as indicated by the present results, is irritable bowel syndrome. Irritable bowel syndrome is characterized by an increase in tone and spasticity of the gastrointestinal tract, most notably the lower bowel (Thompson, 1993). Antimuscarinic compounds (e.g., belladonna alkaloids) are often prescribed for treatment of irritable bowel syndrome; however, the effective dose range for these compounds is near the maximally tolerated dose resulting in side effects typically associated with anticholinergic treatment. As a result patient compliance in long-term treatment is often poor. Thus, alternative approaches for treatment of this condition, and other irritative

conditions of the bowel (e.g., irritable colon, spastic colon, mucous colitis, acute enterocolitis, diverticulitis, and dysenteries), should focus on non-anticholinergic mechanisms for treatment. The present evidence for a role of the σ_2 site in the mediation of ileal function suggests a novel therapeutic target which may not suffer from the accompaniment of the multitude of side-effects associated with anticholinergic compounds.

In summary, the present results demonstrate the first bioassay sensitive to compounds with affinity for σ_2 sites, providing the first means by which elucidation into the functional role of σ_2 sites can be achieved. Further, the results demonstrate the necessity to take into account the subtype specificity of σ site ligands when using them as investigative tools for research into the numerous disorders in which σ related processes have been implicated to play a role. Finally, these results demonstrate an immediate application with regard to irritable bowel syndrome.

Acknowledgements

The authors wish to thank Drs. Roy D. Simmons, Jerry A. Miller and Rae R. Matsumoto for their helpful discussions and comments during the preparation of this paper and Dr. Miller for his help with the construction of Fig. 1.

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